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14. ABSTRACT
Mild traumatic brain injury may affect as many as 400,000 soldiers returning from recent wars in Afghanistan and Iraq. To determine how shockwaves directly affect neurons, we used a biolostic gene gun to deliver shockwaves to cultured hippocampal or cortical neurons. These cultured cells form abundant synapses in vitro, and after a 24-48 hour period of incubation following shockwave exposure, immunocytochemical labeling showed a significant reduction in synaptic densities in shockwave-exposed cultures compared to control cultures. While the molecular

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Report Title

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List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

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Title: Shockwaves Cause Synaptic Degeneration in Cultured Neurons

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Matthew T. Ferenc, Raul Radovitzky, Cameron R. Bass, Garrett Wood, Matthew Panzer, Heng-Ye Man, and James O. Deshler.

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Graduate Students

NAME	PERCENT_SUPPORTED
Matthew Ferenc	1.00
FTE Equivalent:	**1.00**
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Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
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NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
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NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

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Shockwaves Cause Synaptic Degeneration in Cultured Neurons

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Online Abstract

Mild traumatic brain injury may affect as many as 400,000 soldiers returning from recent wars in Afghanistan and Iraq. To determine how shockwaves directly affect neurons, we used a biolostic gene gun to deliver shockwaves to cultured hippocampal or cortical neurons. These cultured cells form abundant synapses *in vitro*, and after a 24-48 hour period of incubation following shockwave exposure, immunocytochemical labeling showed a significant reduction in synaptic densities in shockwave-exposed cultures compared to control cultures. While the molecular mechanism responsible for this phenomenon is not known, these results suggest that shockwaves emanating from explosive devices may specifically affect synaptic plasticity in the brain.

Key Words:

Mild Traumatic Brain Injury, mTBI, synaptic plasticity, shockwave, neuron, biolostic, gene gun, synapse, degeneration, pressure wave

Approximately twenty percent of the 1.6 million soldiers deployed to the wars in Iraq and Afghanistan have suffered a mild Traumatic Brain Injury (mTBI). This injury is thought to be caused by shockwaves emanating from improvised explosive devices (IEDs). Symptoms can include short term memory loss, cognitive disorders, anxiety, and/or emotional irregularities, but no physiological damage is apparent. Diagnoses are made by assessing a patient's psychological profile and combat history. Since a wide range of psychological and emotional disorders, but not physical damage, are associated with mTBI, a report by the RAND corporation referred to this collective set of symptoms as "The Invisible Wounds of War" (*1*).

It is well documented that high overpressure shockwaves can lead to contusional hemorrhage and cell death in the brain (*2, 3*). However, the cellular etiology behind mTBI induced by relatively low overpressure shockwaves in the brain is poorly understood since no evidence of cell death or other forms of severe tissue damage has been detected in mTBI patients. Since synaptic plasticity has been implicated in multiple aspects of higher order brain function (*4*), we considered that a more subtle form of sub-cellular damage involving synaptic plasticity may be at the root of mTBI. We hypothesized that sub-lethal pressure waves derived from exploding IEDs specifically damage synapses, but not other parts of neuronal cells. Synapses are dynamic subcellular structures that both store and transmit information processed by the brain. Thus, if synapses are damaged by shockwaves one would expect the brain to malfunction, even if other components of neurons (*eg.* cell body, dendrites, axons) remain intact.

Two recent observations contributed to this hypothesis. First, when the sub-cellular trafficking machinery that is required for delivering proteins to the axonal and dendritic processes is disrupted, the first indication of neuronal malfunction is the reduction in the number of synapses along the dendrites of the cell (*5*). Secondly, this cellular trafficking machinery is known to be limited by the energy state of the cell in other highly polarized cell types (*6*). Thus, if sub-lethal shockwaves reduce the energy state of neurons, for example by damaging mitochondria or permeabilizing the plasma membrane, it is likely that synaptic degeneration might be the first sign of neuronal damage.

To test whether shockwaves specifically affect synaptic densities of mammalian neurons of the central nervous system we delivered shockwaves to cultures of cortical or hippocampal rat neurons using a biolistic gene gun (See Methods in Supplement). Biolistic gene guns utilize high helium pressures to generate supersonic shockwaves that can be used to deliver DNA-coated microspheres to neurons and other cell types (*7*). In these experiments the revolver-like cylinder was left empty such the neurons were shot only with shock/pressure waves. Input helium pressures varied initially from 50 to 600 pounds per square inch (psi) corresponding to applied peak reflected pressure of 0.03-15 psi at the sample (See Supplement). At input pressures above 400 psi many neurons were killed. At input pressures below 350 psi, overall cell morphologies and viabilities were virtually identical in cultures exposed to shockwaves and control mock-shot cells, similar to previous findings (*8*). At an input pressure of 300 psi, the gene gun delivers a peak overpressure of about 2.4 psi at the surface of the cell culture and peak overpressures increase linearly with input Helium pressures (Figure S1). After shockwave exposure, the neurons were cultured for 24 hours and synapses were visualized using

immunocytochemical methods. Neurons exposed to shockwaves generated with either 200 psi or 300 psi input pressures showed a significant and similar reduction in the number of synapses present along the dendritic shafts of either hippocampal (Figure 1) or cortical (Figure S2) neurons. At input helium pressures of 50 to 100 psi synaptic densities appeared to be unaffected, whereas at 150 psi the results were somewhat variable (data not shown). The pressures delivered to the neuronal cultures in this study are about one tenth to one half of the internal pressures detected in the brains of anesthetized swines subjected to strong, but sub-lethal blast waves (*9*). Since it is likely that myelinated neurons within the brain are more resistant to environmental insults than the cultured neurons studied here, additional experiments will be required to measure blast intensity levels that affect synaptic plasticity in brain tissue and also to determine whether synaptic degeneration *per se* plays a prominent etiological role in mTBI.

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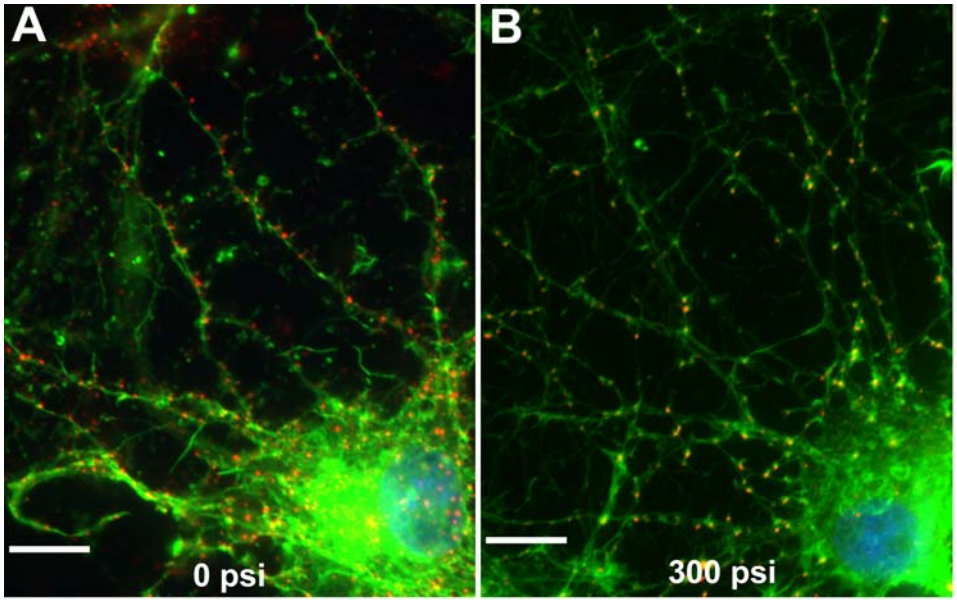
Figure Legend

Figure 1. Reduced synaptic densities in cultured hippocampal neurons exposed to shockwaves. Rat hippocampal neurons cultured for 14 days *in vitro* (DIV) were exposed to a shockwave using a biolistic gene gun with either 200 or 300 pounds per square inch (psi) input Helium pressures. The cells were then cultured for an additional 24 hours, fixed, and stained for the actin cytoskeleton using Alexa-488 phalloidin (green) to label entire cells. A primary monoclonal antibody to post synaptic density protein 95 (PSD95) followed by incubation with an Alexa-555 conjugated secondary antibody (red) was used to label synapses. Nuclear DNA was stained blue with Hoechst dye (A and B). (A) Neuron from a mock-shot culture and (B) a similar neuron from a culture subjected to a 300 psi-generated shockwave. (C) High magnification images of 25μm segments of neuronal processes taken from different neurons chosen arbitrarily from mock-shot cultures or cultures subjected to 200 psi or 300 psi generated shock waves. Segments were identified and selected using only the 488 nm channel such that they were identified without visualizing synapses. After segments of processes were selected, the corresponding PSD labeling patterns were acquired to eliminate bias in the selection procedure. Ten segments from distinct cells within a given culture are shown, and the actin and PSD labeling patterns from the same process are shown adjacent to each other, but are shown in black and white to facilitate visualization of the unprocessed images. (D) A threshold was applied to the images and synapses were quantitated in control cultures and cultures subjected to either 200 psi or 300 psi. Cultures exposed to shockwaves show a ~60 percent decrease in synaptic densities relative to control cultures (**** = $p < 0.0001$). No significant difference was detected between the 200 psi and 300 psi samples. Scale bars = 10 μm.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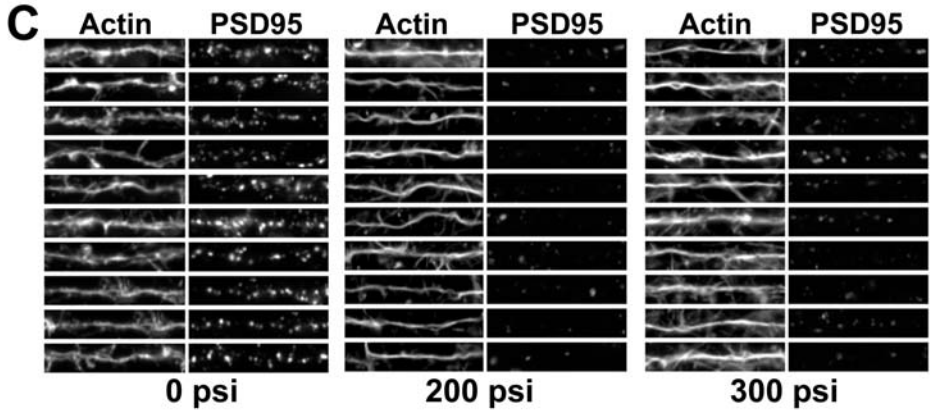
300 psi


C
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PSD95
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Actin
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0 psi
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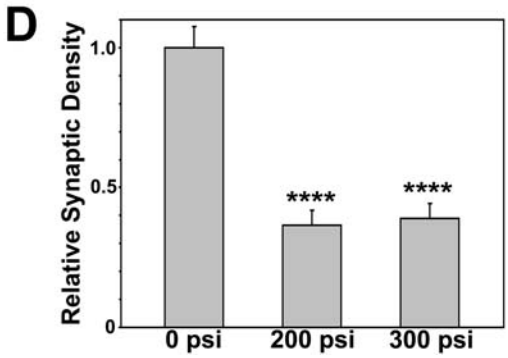
D
Relative Synaptic Density
1.0
0.5
0
****
****
0 psi
200 psi
300 psi

Figure 1

Supplemental Methods and Figures
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Gene Gun Calibration

To determine applied pressure time histories from the Helios Gene Gun (BioRad), incident and reflected pressures were measured. For reflected pressure, the gene gun was fixed rigidly to a stand with the gene gun spacer positioned 2 cm above a flat plate constructed of delrin. A piezoresistive pressure sensor (Endevco Model 8530C) was mounted flush with the plate, coaxial with the center of the gene gun output, in the direction of the flow. Incident pressure was measured by mounting a piezoresistive pressure sensor 2 cm from the end of the gene gun spacer, perpendicular to the flow direction. The sleeve surrounded the gene gun spacer from the base of the gun to 3 cm from the end of the spacer and was open in the direction of the flow.

Gene gun output was measured with helium input pressures of 50, 100, 150, 200, 300, 400, and 500 pounds per square inch (psi) for 9 tests for both reflected and incident pressure. To ensure repeatability, the gene gun was fired 3-4 times at each pressure before measurements were taken. Data were acquired using a multichannel signal conditioner (Vishay Model 2160) and LabVIEW (National Instruments). Incident pressure data was digitized at 500 kHz while stagnation pressure was digitized at 300 kHz. Data were post-filtered at 40 kHz using an 8-pole Butterworth filter. Acquired data was analyzed using MATLAB (Mathworks) to determine peak wave pressure and rise times from 20-80% of peak for incident pressures (Supplemental Figure 1A) as well as reflected pressures and impulses (Supplemental Figure 1B).

Shockwave exposure and cell labeling.

Primary cortical neuronal cultures were prepared from E18 rat embryos as previously described (*1*) and cells were plated at an original density of $6x10^6$ cells/cover slip. Primary hippocampal neuronal cultures were prepared similarly from E18 rat embryos also following a previously established method (*2*) and plated at a density of $6x10^6$ cells/cover slip. All cells were cultured on cover slips in 60 mm culture dishes. After 14 days of culturing cells *in vitro*, most of the culture media was removed from an individual well leaving approximately 100ul to cover the cells. The gene gun was placed directly above the coverslip with the barrel guard placed 5-8 mm above the cells. The gun was discharged and the media was immediately replaced back onto the cells such that the cells were uncovered for no more than 30 seconds. In some experiments, the liquid was left in place and covered with plastic wrap during the shooting, and similar reductions synaptic densities were observed (data not shown).

After exposure to shockwaves, the cells were cultured for an addition 24 or 48 hours. Prior to fixation cells were briefly washed in cold 1X ACSF (~750µl) three times. Any removal or addition of liquid was done at the side of the well and not directly on any coverslip to avoid physically damaging cells. Cells were then fixed in a 4% PFA/surcrose solution (pH 7.4) for 10minutes at room temperature. After fixation cells were washed three times in cold 1X ACSF. Cells were then permeabilized with 500ul of 0.3% TritonX in 1X PBS for 10 minutes at room temperature. Cells were then washed three times in cold 1X ACSF and blocked for 1hour at room temperature in 10 percent goat serum (in 1X PBS). Primary antibody was then added. For PSD95 a monoclonal antibody (Catalog # AB9708, Millipore) was used at a dilution of 1:2000 and incubation was carried out for 3 hours at 4 degrees C. For GluR1, a polyclonal antibody generated from the C-term fragment was used as previously described (*2*) at a 1:1000, and similar results were obtained with a commercially available version of the same antibody (Millipore #AB1504). An anti-alpha tubulin monoclonal antibody (Sigma T9026) was used at a 1/3000 dilution and incubation was carried out for one hour at room temperature. Secondary antibodies (A11001 and A21422, Invitrogen) were used at a 1/3000 dilution and incubations were carried out for one hour at room temperature. Cells were washed three times in cold 1X ACSF before the addition of secondary antibody as well as Hoescht stain. Phalloidin actin staining was also performed during the secondary antibody labeling. Cells were washed five

times in cold 1X ACSF and then mounted for observation using Prolong Gold Antifade Reagent (Invitrogen cat. # P36930).

Image acquisition and quantitation.

Images were acquired as previously described (*1*). Quantitation of synaptic staining was conducted using ImageJ (version 1.41K). Neuronal processes were selected based solely on cytoskeletal (tubulin or actin) staining patterns from the green channel without examining the red staining patter whatsoever. All processes selected could be traced back to a cell body within the same field of view, and all segments analyzed were selected to be at least 50 microns long with as little background signal as possible. The process was then measured for length and a region of interest (ROI) was drawn around the segment using the Polygon tool with approximately 15microns extra width on either side of the process. This ROI was then saved, and the synaptic markers (PSD96 or GluR1) on the red channel then analyzed. A threshold of 1500 was applied to the red channel, and the Analyze Particles function then counted all particles in the ROI between 0.003-infinite square microns, as well as recording their mean grey value (MGV) and area. This information was imported into MS Excel and used to determine the number of particles per unit of length for that particular process. This was repeated at least 30 times with no more than 2 processes being chosen from any one image. The particles/micron measurements were then averaged and standardized to the 0 psi group for graphing the resulting data.

Supplemental Figure 1:
(A) Incident peak pressures and rise times with increasing input Helium pressures detected at a distance from the gene gun equivalent to that used for exposing neuronal cells to gene gun-induced pressure waves. (B) Reflected pressures and impulses detected with increasing input Helium pressures.

Supplemental Figure 2:
The staining pattern of GluR1 (red), a subunit of the major class of AMPA receptors found in the brain (approximately 80% of all synapses) decreases significantly in cultured neurons subjected to a sockwave. (A) 15 segments of processes (20μm each) chosen based on tubulin staining (green) from cultured cortical neurons subjected to either 0 psi (control) or 200 psi at 15DIV. GluR1 is red, scale bar is 5μm. (B) Quantitation of GluR1 staining shows a statistically significant decrease between the control neurons (0psi) and those subjected to a 200psi shockwave ($p<0.0094$).

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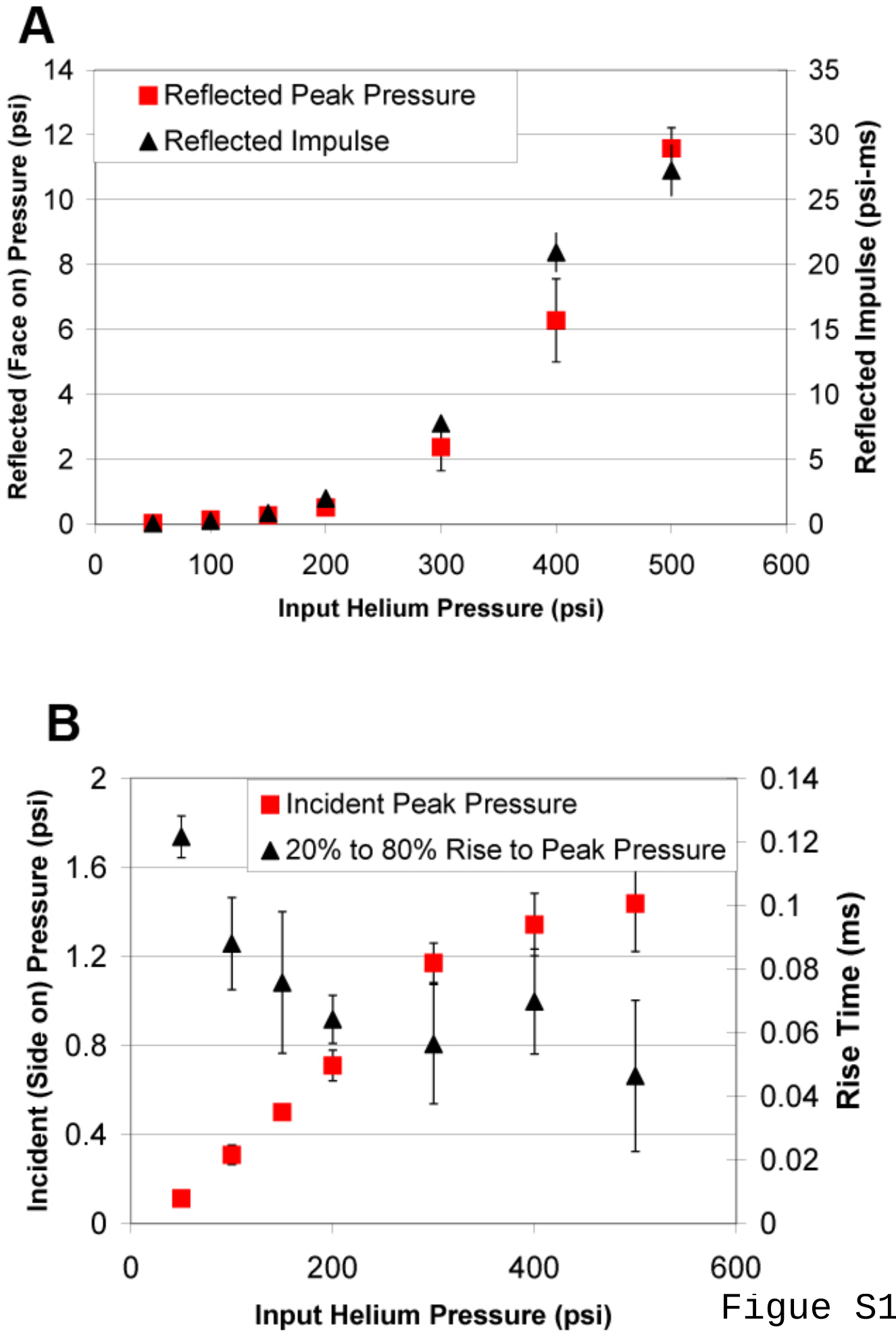
A
Reflected Peak Pressure
Reflected Impulse
Reflected (Face on) Pressure (psi)
Reflected Impulse (psi-ms)
Input Helium Pressure (psi)
0
2
4
6
8
10
12
14
0
5
10
15
20
25
30
35
0
100
200
300
400
500
600
B
Incident Peak Pressure
20% to 80% Rise to Peak Pressure
Incident (Side on) Pressure (psi)
Rise Time (ms)
Input Helium Pressure (psi)
0
0.4
0.8
1.2
1.6
2
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0
200
400
600

Figue S1

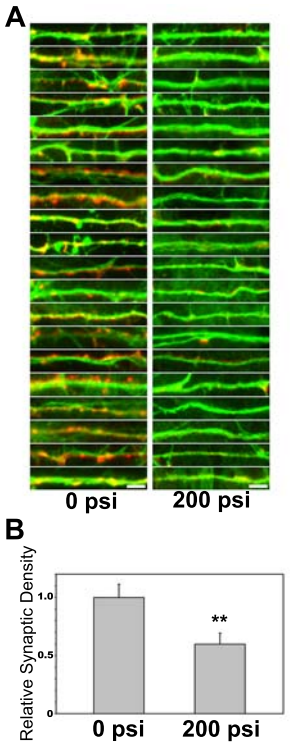
A
0 psi
200 psi
B
Relative Synaptic Density
1.0
0.5
0
**
0 psi
200 psi

Figure S2